Jasminka Brnjas-Kraljević
Greta Pifat-Mrzljak
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Preface

In the fifties of the last century the definition of biophysics arose much dispute among the scientists who were traditional physicists, chemists and biologists by training. As an interdisciplinary science, biophysics shares significant overlap with biochemistry, bioengineering and systems biology, but on the other hand offers a rational language for discussion about a common subject to scientists of different scientific disciplines. Biophysics has gradually erased the frontiers in scientific research by bringing together scientists from different fields of research. Nowadays, it has been widely accepted that the search for new knowledge depends not only on new methods and concepts but also on the interaction within different fields of research. Promoting an interaction between different disciplines in natural sciences and enabling young scientists to be involved in it is the general philosophy behind the Biophysical Summer Schools organized by the Rudjer Bošković Institute, Zagreb, Croatia and the Croatian Biophysical Society every 3 years, since 1981.

The International Summer Schools on Biophysics have a broad scope devoted to the structure-function relationship of biological macromolecules and to mayor biophysical techniques. They are internationally recognized and successfully established under the title "Supramolecular Structure and Function" and are included into the curricula of doctoral studies at distinguished European universities. The intention has remained the same through all the ten Schools – to organize courses which provide advanced training at doctoral or postdoctoral level in biosciences. The Schools have gained reputation for running Discussion Clubs as extra curricular activities, where students would invite their peers to gather around lecturers and discuss various topics of specific interest. The enthusiasm of these discussions is always equally shared by students and lecturers. The contributions presented at the Summer School by prominent lecturers illustrate the principles, concepts and methods of biophysics coupled with molecular biology approaches. Given the considerable diversity of topics it covers, we believe that the book will be of interest to scientists involved in different disciplines, as it was to the audience at the Summer School.

The tenth Summer School, as Master Classes of UNESCO, was supported by UNESCO and could be considered as a part of the mosaic forming the European Research Area (ERA) and the European Higher Education Area (EHEA).

The organizers of the International Summer School on Biophysics hope that the publication of this volume and its distribution within the scientific community will serve towards the objectives of expanding, sharing and providing easy access to scientific knowledge in the field of biophysics.

The support to the School by IUPAB and EBSA reflects the international and European interest to bring together scientists of different profiles from all over the world. The national financial supporters were the Ministry of Science, Education and Sport of the Republic of Croatia, the Croatian Academy of Sciences and Arts, The Adris Foundation and The National Foundation for Science, Higher Education and Technological Development of the Republic of Croatia whose substantial support enabled the participation of young scientists from Croatia.

This volume will inform the broader scientific community on the profile of the Summer School and new biophysical achievements, but the most valuable outcome of the tenth School is the exchange of knowledge and friendships established between lecturers and participants in the pleasant atmosphere of the Crveni otok near Rovinj, Croatia.

Zagreb, Croatia

Greta Pifat-Mrzljak
Director of the Schools

The *spiritus movens* of all ten Schools during the period of 30 years was professor Greta Pifat-Mrzljak. Unofortunately, she passed away December 11, 2009. Till the last she was involved in organization and preparation of the School and this proceeding. The tradition, she established, was to present the School with book of selected lectures held on School by distinguished lectures. The intention was to acquaint the brooder scientific communion with the hot subjects in biophysical research. The result of 30 years devotement is the serial of ten books "Supramolecular Structure and Function" as a nice history of biophysical research development and a great help in education of young scientist in that field. Detailed information about Schools and books can be found on web site of the School

http://www.irb.hr/events/confpages/biophysics/

For the outstanding record of accomplishments and leadership of the triennial international summer schools and textbooks on Supramolecular Structure and Function prof. Greta Pifat-Mrzljak was presented the 2010 Emily M. Gray Award by The Biophysical Society.

Zagreb, Croatia

Jasminka Brnjas-Kraljević
on behalf of Organizing Committee

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Fluorescence Correlation Spectroscopy: Principles and Developments

Sergey Ivanchenko and Don C. Lamb

Abstract Twenty years ago, fluorescence measurements at low concentrations were difficult due to the weak fluorescence signal and intrinsic fluctuations of the sample. With the development of FCS and its implementation on a confocal microscope, it is possible to use the inherent fluctuations to gain information over the concentration, molecular brightness, microscopic rate constants for reactions and mobility of the measured sample. In recent years, there has been a strong increase in the development and application of fluctuation methods. With pulsed interleaved excitation, stoichiometry information can be obtained and spectral cross-talk can be eliminated from FCCS experiments. An elegant implementation of two-focus FCS has also been introduced to allow absolute measurements of diffusion coefficient without precise knowledge of the psf of the microscope and is less sensitive to the laser excitation intensity and saturation effects. Scanning methods such as Scanning FCS and RICS increase the effective volume, which is advantageous for live-cell measurements where diffusion is slow and photobleaching is a problem. In this article, describe the basics of FCS and its limitations as well as a short discussion of a handful of emerging techniques. There are still many other equally interesting applications of fluorescence fluctuation spectroscopy that we have not been able to touch upon. And, if the past is any indication of the future, there will be a number of novel fluorescence fluctuation spectroscopy methods emerging in the near future.

Keywords Fluorescence correlation spectroscopy (FCS) · ACF · ALEX · ccRISC · FRET · PIE

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Abbreviations

2fFCS	Two-focus fluorescence correlation spectroscopy
ACF	Autocorrelation function
ALEX	Alternating laser excitation
CCF	Cross-correlation function
ccRISC	Cross-correlation raster image correlation spectroscopy
FCS	Fluorescence correlation spectroscopy
FCCS	Fluorescence cross-correlation spectroscopy
FRET	Förster resonance energy transfer
PIE	Pulsed interleaved excitation
RISC	Raster image correlation spectroscopy

1 Introduction

Approximately 100 years ago, the first fluorescence microscopes were built (Heimstadt 1911, Reichert 1911, Lehman 1913). Fluorescence has many advantages for investigating biological systems; for example, cells are typically transparent to visible light and fluorescence experiments can be performed without direct contact with the sample. Often, background fluorescence is low and fluorescence microscopy can be performed with high contrast. In addition, the fluorescence signal can be detected with high sensitivity, as single-photon counting detectors with high quantum yield are currently available. It is possible to perform fluorescence experiments over a broad range of concentrations that extends down to the single molecule scale due to advances in detector sensitivity, aberration free optics and the development of stable light sources. When performing experiments with molecules in solution at low concentrations (e.g. in a cuvette or in the focus of a confocal microscope), the detected fluorescence signal is noisy. This noise does not depend on the quality of the detectors or the stability of the excitation sources, but arises from fluctuations in the number of fluorescent molecules in the observation volume. Due to laws of thermodynamics, the number of molecules in the detection volume constantly fluctuates, giving rise to fluctuations in the detected fluorescence signal. Thermodynamic fluctuations were first observed experimentally with gold beads already in 1911 (Svedberg and Inouye 1911), verifying the predications of Einstein (Einstein 1905) and von Smoluchowski (von Smoluchowski 1906). Fluctuations contain interesting dynamic information regarding the sample and can be extracted from the data with the appropriate methods. To this end, a correlation approach has been developed. Events that are correlated, such as the detection of multiple photons from the same molecule traversing the observation volume, will show up in a correlation analysis. Thereby, information regarding the mobility and average number of the fluorescent molecules in the observation volume can be determined.

Fluorescence Correlation Spectroscopy (FCS) was first performed by Madge et al. (1972). In the first years, the group published three seminal works on FCS including the theory for freely diffusing particles, the unimolecular and bimolecular reactions (Elson and Magde 1974), experimental realization of the method

(Magde et al. 1974) and the expansion of FCS for systems under flow (Magde et al. 1978). In the early days of FCS, Ehrenberg and Rigler expanded the theory of FCS to describe rotational Brownian motion (Ehrenberg and Rigler 1974, 1976). A thorough description of the error analysis involved in FCS was published by Koppel (1974). Initially, FCS required long measurement times and had a low signal-to-noise ratio due to the low detection efficiencies, high background and large volumes used in the initial systems. A significant improvement came when FCS was combined with confocal microscopy that was first implemented by Koppel and coworkers (1976) and later championed by Rigler and coworkers in the 1990s (Rigler et al. 1993, Eigen and Rigler 1994, Widengren et al. 1994, 1995).

Today, FCS is widely applied in a broad number of disciplines including physics, chemistry, biology, biophysics, biochemistry and medicine. The apparatus is commercially available and an FCS signal is easy to obtain. Anything that alters the fluorescence intensity in a correlated way will show up in an FCS measurement. This is one of the advantages of FCS but is also an aspect that requires caution. This is especially true for measurements of slowly diffusion particles or for FCS measurements in living cells where many external variables such as mechanical vibrations or oscillations in laser intensity can contribute to the correlation function.

2 Principles of FCS

2.1 What Is FCS?

FCS has been used to measure a large number of phenomena including translational diffusion (Magde et al. 1974), rotational diffusion (Ehrenberg and Rigler 1974, Aragón and Pecora 1976, Kask et al. 1989), triplet-state dynamics (Widengren et al. 1994, 1995), chemical reactions (Magde et al. 1974, Magde 1976, Rauer et al. 1996, Lamb et al. 2000a, Bismuto et al. 2001) and conformational fluctuations (Bonnet et al. 1998, Torres and Levitus 2007). The fundamental process upon which FCS is based is the non-stochastic nature of the fluorescence photons being detected. For example, in translational diffusion, an increase in the number of detected photons is observed when a fluorescent molecule enters the observation volume and again the signal decreases when the molecule diffuses away. This correlation is extracted using a correlation analysis. The typical FCS experiment is based upon two assumptions: (1) the system is stationary, meaning that the average values of the phenomena being measured do not change with time and (2) the system is ergodic. Hence, every sizable sampling of the process is representative of the whole. The details of what FCS is and how it works are discussed below.

2.1.1 Fluorescence

FCS is based upon fluorescence. Fluorescence is the property of a molecule to emit light upon returning to the ground state from the lowest level of the singlet excited state after optical excitation (Fig. 1a). Molecules that can emit light upon such an

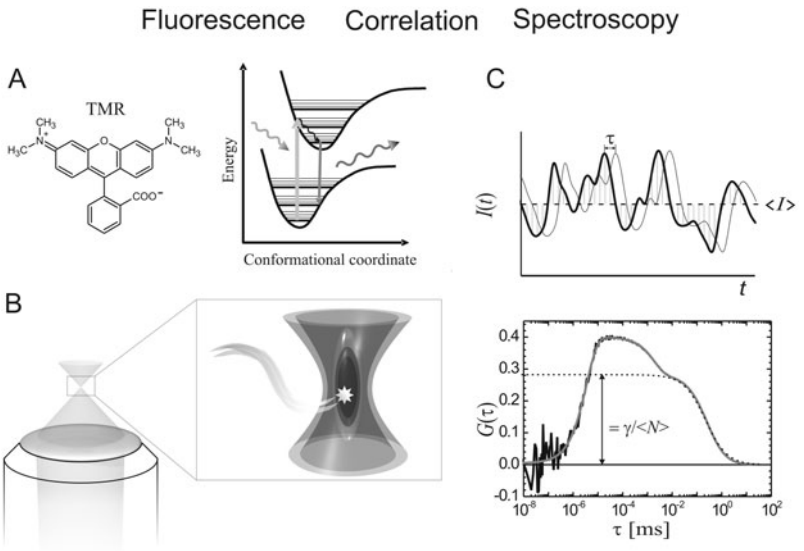

Fig. 1 Fluorescence Correlation Spectroscopy. FCS is based upon fluorescence. **A** The chemical structure of a typical fluorescent molecule, tetramethylrhodamine, along with the Franck-Condon diagram of the electronic transition. **B** A schematic diagram of the focus of a confocal microscope with a blow up of the excitation (*cone shaped*) and detection (*ellipsoidal*) volumes. A molecule diffusing through the confocal volume is shown as a *star*. **C** The self-similarity of the fluorescence time series (*upper panel*) is reflected in its autocorrelation function (*lower panel*). ACF from a diffusing molecule shows several processes that occur during its passage through the confocal volume; anti-bunching, triplet-state excitation and translational diffusion. The amplitude of the translation diffusion component is inversely proportional to the total number of fluorescent molecules in the confocal volume. The plot is adopted from (Felekyan et al. 2005)

electronic transition are called fluorophores (e.g. tetramethylrhodamine, Fig. 1a). The fluorophores that are typically used contain an extended π-electron conjugated system in which electrons can move freely. Such conjugated π-electron systems have a large cross section for absorption of visible light and upon absorption of a photon, an electron is transferred into an electronic excited state. The electron relaxes back to the ground state within nanoseconds, giving up the absorbed energy in a form of either a photon or a phonon. Different fluorophores have different fluorescent properties such as different excitation and emission spectra, and these differences can be exploited to investigate multiple species and their interactions simultaneously.

Typically, FCS is performed on a fluorescence confocal microscope[1]. See e.g. (Webb 1996) for a review on confocal microscopy. The focal size of the confocal microscope is limited via diffraction to roughly 1 fL. The optical response of the microscope to a point source at the center of the focus is referred to as the

[1]It is also possible to perform FCS using Total Internal Reflection Excitation, but a description of this method is beyond the scope of this publication. For details see Thompson et al. (1981).

point-spread-function (psf) or observation volume and is approximated by a three-dimensional Gaussian with different lateral and axial dimensions (Fig. 1b). The observation volume is the overlap between the excitation, sample and detection volumes and is given by:

$$W(\mathbf{r}) = I_0(0) \exp\left[-\frac{2\left(x^2+y^2\right)}{w_r^2} - \frac{2z^2}{w_z^2}\right] \tag{1}$$

where w_r and w_z are the radial and axial distance from the center of the psf to where the intensity has decreased by $1/e^2$. If we assume that we have freely diffusing, non-interacting particles that do not undergo photophysical effects, we can determine the total measured fluorescence intensity by the position of the particles as a function of time. The fluorescence signal is then given by:

$$F(t) = \kappa\sigma\phi \int d\mathbf{r}\; W(\mathbf{r})C(\mathbf{r},t), \tag{2}$$

where κ is the overall detection efficiency of the system, σ is the absorption cross section at the wavelength of excitation, ϕ is the fluorescence quantum yield of the fluorophore and $C(\mathbf{r},t)$ represents the concentration of particles at position $\mathbf{r}$ and time t. The product:

$$\varepsilon = \kappa\sigma\phi W\,(\mathbf{0}), \tag{3}$$

yields the molecular brightness of the fluorophore at the center of the psf.

Other methods exist that utilize the information obtainable from equilibrium fluctuations of the sample that do not rely on fluorescence. Dynamic light scattering, for example, detects photons scattered from a sample, which are correlated to determine the translational diffusion coefficient of molecules. However, FCS is more sensitive and can be performed at lower concentrations than dynamic light scattering.

2.1.2 Correlation

The heart of FCS is the correlation analysis. It is the correlation function that allows us to extract information regarding the fluctuations. The temporal autocorrelation function (ACF), also referred to as the normalized second-order autocorrelation function, is defined as:

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle - \langle F(t)\rangle^2}{\langle F(t)\rangle^2} = \frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2} \tag{4}$$

where $\langle\rangle$ refers to the time averaged value and $\delta F(t) = F(t) - \langle F(t)\rangle$. The denominator renormalizes the ACF to the average fluorescence intensity. The ACF measures the self-similarity of the fluorescence intensity time series as a function of the delay time τ, also referred to as the correlation time (Fig. 1c). At zero delay, the amplitude of the ACF is given by:

$$G(0) = \frac{\langle \delta F(0) \delta F(0) \rangle}{\langle F \rangle^2} = \frac{\sum_{i=1}^{\ell} (F(t_i) - \langle F \rangle)^2 \Big/ \ell}{\left(\sum_{i=1}^{\ell} F(t_i) \Big/ \ell \right)^2} = \frac{\sigma^2}{\mu^2} \tag{5}$$

where σ^2 is the variance of the time series and μ the average fluorescence intensity. Due to the definition of the ACF (Eq. 4), it has a maximum value at $G(0)$. This can be seen from the fact that when there is no shift, (i.e. $\tau = 0$), the maximum value of $\delta F(t)$ will be multiplied by itself summed with the second maximum value of $\delta F(t)$ multiplied by itself and so on. All points will add constructively as $\delta F(t)^2 > 0$. For non-conserved, non-periodic signals, $G(\tau) \to 0$ as $\tau \to \infty$.

Assuming a 3D Gaussian observation volume for $W(\mathbf{r})$ (Eq. 1), using Eq. (2) for the fluorescence intensity, the ACF (Eq. 4) can be solved analytically and is given by:

$$G_D(N, D, \tau) = \frac{\gamma}{\langle N \rangle} \left(\frac{1}{1 + \tau/\tau_D} \right) \left(\frac{1}{1 + (w_r/w_z)^2 \tau/\tau_D} \right)^{1/2} \tag{6}$$

$$\text{where} \quad \tau_\text{D} = \frac{w_r^2}{4D} \text{ or } \tau_\text{D} = \frac{w_r^2}{8D} \tag{7}$$

for one- and two-photon excitation respectively and γ is a factor that depends on the geometry of the observation volume ($\gamma = 2^{-3/2}$ for a 3D Gaussian). See Section 2.2 for more details regarding the γ factor.

In literature, a second definition of the ACF is also used:

$$g(\tau) = \frac{\langle F(t) F(t + \tau) \rangle}{\langle F(t) \rangle^2}. \tag{8}$$

For a 3D Gaussian observation volume, the ACF in this form is given by:

$$g(\tau) = 1 + \frac{\gamma}{\langle N \rangle} \left(\frac{1}{1 + \tau/\tau_D} \right) \left(\frac{1}{1 + (w_r/w_z)^2 \tau/\tau_D} \right)^{1/2}. \tag{9}$$

In this representation, the ACF is proportional to the probability of detecting a photon at the time τ, given that a photon was detected at $\tau = 0$. At long times, when no correlation is observable, the probability of detecting a photon is constant and equal to the random possibility of a second photon being detected depending on the average count rate.

2.1.3 Spectroscopy

The remaining term in the name of the method is spectroscopy. In particular, we are performing spectroscopy on the fluctuations and relate the properties of the fluctuations to properties of the fluorescent molecules. The ACF for Rhodamine 110 freely

diffusing in solution is shown at the bottom of Fig. 1c. Several processes influence the shape of the curve. At shorter times ($\sim 10^{-9}$ s), the lack of correlation is caused by a non-zero delay between the absorption and emission of a photon. During this delay, no further absorption-emission events are possible, which leads to loss of correlation. This rising edge of the ACF is referred to as the antibunching term and is typically not recorded in most experiments. However, the early times are useful for precise measurements of polarization anisotropies of fluorophores.

The maximum of the ACF (Fig. 1c) occurs around 10^{-5} ms and decays in two characteristic steps. The microsecond decay is typically related to relaxation of the fluorophore from the triplet state and its amplitude depends on excitation power. The amplitude increases with increasing power. For larger molecules such as proteins, other processes like rotation also contribute to the ACF on this timescale. Measurements at such short times are difficult due to distortions introduced by the detector (e.g. detector dead time or detector afterpulsing). To measure the early correlation times, two detectors are used where the light is split equally between the two detectors and cross-correlation of the signal from the two detectors is performed (Brown and Twiss 1956).

The second step in the ACF seen at longer times is caused by translational diffusion and depends on the average time the molecule spends in the observation volume. The average duration of the fluctuations, given by the decay time of the ACF (τ_D), can be related to the translation diffusion coefficient of the fluorescent molecule. From Eq. (7), D can be calculated if ${w_r}^2$ is known or, if the diffusion coefficient is known (for example, $D = 414 \pm 5$ $\mu m^2/s$ for Rhodamine 6G in buffer at 25°C (Muller et al. 2008)), the size of the psf can be determined. As D and ${w_r}^2$ always appears together in Eq. (7), D can only be determined to the accuracy to which ${w_r}^2$ is known. This difficulty can be overcome by bringing an absolute distance into the equation as is the case of two-focus FCS described in Section 3. Fluctuations other than translation diffusion, which occur on different time scales, can also be analyzed with FCS and related to physical parameters such as the triplet-state-lifetime or the microscopic rate coefficients.

The amplitude of the fluctuations also provides relevant information regarding the fluorescent molecules such as the fraction of molecules in the triplet state or information regarding the equilibrium coefficient of a unimolecular reaction. As the ACF is renormalized to the intensity, the amplitude of the fluctuations due to translational diffusion and thus also the corresponding ACF, is inversely proportional to the average number of particles in the observation volume, N (Fig. 1c, bottom panel). To compare the number of particles with the actual concentration in mol/L, the geometric factor γ needs to be considered.

2.2 The Geometrical Factor γ

The difficulty in converting the number of particles in the observation volume into a concentration is determining the size of the observation volume. In the ideal case, the fluorescence intensity emitted from fluorophores inside the observation volume